REMARKS

Claims 85-91 and 93-110 are pending. Claim 92 has been cancelled without prejudice, admission, or disclaimer. Claims 85-91, 93-101, 105, 107, and 108 have been amended, and claims 109 and 110 have been added. Support for amended claim 85 and claims depending therefrom is found generally throughout the entirety of the application as filed and in particular, for example, at page 16, lines 24-26, page 17, lines 5-6, and page 20, line 17. Support for new claims 109 and 110 is found, for example, at page 21, line 10. Consideration of the currently listed claims as well as the remarks that follow is respectfully requested.

Applicant inadvertently omitted copies of two references listed on form 1449 filed 13 February 2004. Applicant acknowledges the Examiner's comments, and apologizes for this oversight. Enclosed herewith are copies of the Agrawal and Bailey references, as well as a new form 1449 listing these two references. Acknowledgment that these references have been considered by the Examiner is respectfully requested.

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Claim Rejections

Rejections Under 35 U.S.C. §112, First Paragraph – Written Description

Claims 85-93, 97-106, and 108 stand rejected as lacking written description support in the application as filed. The Office Action expresses that written description support is lacking for other than titratable lipids having a pK_a range as set out in claim 1 of U.S. Patent 6,858,225, or lipids containing protonatable or deprotonatable groups having a pK_a as set out in claim 1 of U.S. Patent 6,287,591. Preliminarily, Applicant points out that claim 92 has been cancelled.

Without addressing the propriety of the rejection, in the interest of advancing prosecution, Applicant has amended claim 85 to insert language referred to by the Examiner which further characterizes the amino lipid of the nucleic acid-lipid particle. In particular, claim 85 as currently amended recites that the lipid layer surrounding and encapsulating nucleic acid of the nucleic acid-lipid particle comprises (i) an amino lipid comprising an amino group having a pK_a of from 4 to 11, and (ii) a polyethyleneglycol-diacylglycerol (PEG-DAG) conjugate. Support for currently amended claim 85 is found generally throughout the entirety of the application as filed and in particular, for example, at page 16, lines 24-26, page 17, lines 5-6, and page 20, line 17.

In respect of claims 103 and 104, the Office Action expresses that support is not found in the specification for the recitation that nucleic acid is not substantially degraded after exposure of the claimed particles to either nuclease (claim 103) or serum (claim 104) for 20 minutes at 37°C. The Office Action further contends that Example 10 provides inadequate support for these claims, stating at page 3: "A time of 20 minutes is not found in the specification." Applicant respectfully disagrees with this allegation and traverses the rejection.

Example 10 (page 70) of the present application discloses the results of two different experimental schemes, the first using S1 nuclease, and the second using serum, to assay the

stabilizing effects of encapsulation on phosphodiester and phosphorothioate oligonucleotides. The application states:

> "Free phosphorothioate oligodeoxynucleotide showed significant breakdown in serum within 30 minutes, however encapsulated phosphorothioate oligodeoxynucleotide did not show any sign of degradation even after 24h incubation in serum. In vivo data agrees with these findings, showing no sign of degradation of the encapsulated phosphorothioate antisense until 8h.
>
> As a positive control, the free phosphodiester and phosphorothioate antisense were subjected to very potent levels of S1 nuclease (100U/50μg) (1U of S1 nuclease will digest 1μg DNA per minute at 37°C). The enzyme completely digested the free phosphodiester and phosphorothioate within seconds after its addition. The encapsulated phosphodiester under the same conditions was over 90% intact at 24h, and the encapsulated phosphorothioate was fully intact at 24h."

Concerning the requirements for written description support, the M.P.E.P. at §2163.02 instructs: "The subject matter of the claim need not be described literally (i.e., using the same terms or in haec verba) in order for the disclosure to satisfy the description requirement." Despite the lack of an explicit recitation of "20 minutes" (claim 103) or "30 minutes" (claim 104), the specification clearly discloses nucleic acid-lipid particles wherein the nucleic acid is not substantially degraded after exposure to serum or a nuclease at 37°C for 20 minutes or 30 minutes, as would be recognized by one of ordinary skill in the art. Accordingly, Applicant submits that the rejection of claims 103 and 104 as lacking written description support under 35 U.S.C. §112, first paragraph, is improper and should be withdrawn.

Applicant submits that the current amendment addresses and overcomes the outstanding bases set forth for rejection of claims 85-91, 93, 97-106, and 108 under 35 U.S.C. §112, first paragraph, and that the currently listed claims find written description support in the application as filed. Accordingly, Applicant respectfully requests withdrawal of the rejection, and allowance of the claims.

Rejections Under 35 U.S.C. §112, Second Paragraph

Claims 89-93, 95, 96, and 107 stand rejected under 35 U.S.C. §112 second paragraph. The Office Action alleges that claims 89-93 are indefinite for expressing quantities of particular lipid components in the claimed nucleic acid-lipid particles as percentages of total lipid without reciting all lipids that form the particles. Applicant respectfully traverses the rejection. Preliminarily, Applicant points out that claim 92 has been cancelled.

The essential inquiry with respect to 35 U.S.C. §112, second paragraph, is whether the claims set out and circumscribe a particular subject matter with a reasonable degree of clarity and particularity from the point of view of one possessing an ordinary level of skill in the pertinent art at the

time the invention was made. Further, the definiteness of claim language must be analyzed in light of the content of the application (M.P.E.P. §2173.02).

Claims 89-91 and 93 are not directed to mere collections of cholesterol, amino lipid, and PEG-DAG. Rather, the specification and claim 85, from which claims 89-91 and 93 depend, make clear that these recited elements come together in the context of the nucleic acid-lipid particles of the invention. Accordingly, the various particular components of the claimed compositions are called for in relative amounts, expressed as percentages of the total lipid of the nucleic acid-lipid particles. The present specification, including the experimental section, uses percentages of total lipid to describe relative molar amounts of various components in the nucleic acid-lipid particles of the invention. For example, see page 20, lines 11-12, and page 43, lines 26-27. Applicant respectfully asserts that this is an appropriate and acceptable means of conveying the amounts of the particular components called for within the scope of the claimed nucleic acid-lipid compositions. Indeed, Applicant's assertion is supported by conventional usage of such language in the art. For example, see U.S. Patent Nos. 6,465,008 and 6,296,870, which claim liposome compositions and express the amounts of particular components as percentages of the total without explicitly reciting the total lipid composition.

As stated in M.P.E.P. §2173.02: "In reviewing a claim for compliance with 35 U.S.C. 112, second paragraph, the examiner must consider the claim as a whole to determine whether the claim apprises one of ordinary skill in the art of its scope and, therefore, serves the notice function required by 35 U.S.C. 112, second paragraph, by providing clear warning to others as to what constitutes infringement of the patent." Applicant asserts that given a particular nucleic acid-lipid particle comprising a PEG-DAG conjugate and an amino lipid comprising an amino group having a pK_a of from 4 to 11, which is the context set by the present claims, one possessing an ordinary level of skill in the art could readily determine whether the particle was composed of, for example, between 35-55% cholesterol. Accordingly, Applicant submits that claims 89-91 and 93 set out and circumscribe the present subject matter with a reasonable degree of clarity and particularity such that the skilled artisan can readily determine the full scope of the claimed subject matter. Accordingly, Applicant respectfully requests withdrawal of the rejection and allowance of claims 89-91 and 93.

With respect to claims 95, 96, and 107, the Office Action points out that the claims reiterate elements recited in claims from which they depend. Applicant has amended claims 85, 95, 96, and 107, and submits that the currently amended claims overcome the rejection. Withdrawal of the rejection and allowance of the currently amended claims is respectfully requested.

Rejections Under 35 U.S.C. §103

Claims 85-93, 97-106, and 108 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Wheeler et al. (U.S. Patent No. 6,586,410), in view of Collins (U.S. Patent No. 6,355,267), Cullis et al. (U.S. Patent No. 6,417,326), and Wheeler et al. (WO 96/40964). Additionally, claims 94-96, and 107 stand rejected under 35 U.S.C. §103(a) as being unpatentable in further view of Unger et al. (U.S. Patent 6,143,276). Applicant respectfully traverses.

As a preliminary matter, Applicant points out that claim 92 has been cancelled and that claim 85 has been amended to insert language further characterizing the amino lipid component of the claimed nucleic acid-lipid particle. In particular, claim 85 as currently amended is directed to a nucleic acid-lipid particle comprising a lipid layer surrounding and encapsulating a central region containing a polyanionic nucleic acid, wherein the lipid layer comprises (i) an amino lipid comprising an amino group having a pK_a of from 4 to 11, and (ii) a polyethyleneglycol-diacylglycerol (PEG-DAG) conjugate.

In order to establish a prima facie case of obviousness, the prior art must teach or suggest every limitation of the rejected claims.

Wheeler et al. (U.S. Patent No. 6,586,410; and WO 96/40964) disclose lipid-nucleic acid particles comprising cationic lipids which carry a net positive charge at physiological pH. The compositions are preferably formed by removal of solubilizing component, such as a detergent or organic solvent. Wheeler et al. further teach that the compositions may include PEG-lipids, such as PEG-ceramide.

Collins (U.S. Patent No. 6,355,267) discloses methods for forming liposomes without the use of detergent or organic solvent which involve cycles of freezing and thawing, as well as liposomes so formed. One lipid suggested for use in the liposomes is diacylglycerol.

Cullis et al. (U.S. Patent No. 6,417,326) disclose fusogenic liposomes comprising lipids capable of adopting a non-lamellar phase and reversibly associating with a bilayer stabilizing component. Suggested bilayer stabilizing components include PEG conjugated to ceramide. Additional components suggested for use in the fusogenic liposomes include lipopeptides formed from diacylglycerol.

Unger et al. (U.S. Patent No. 6,143,276) disclose vesicles containing gaseous precursors. The use of DSPC in the preparation of lipid vesicles is disclosed.

Applicant submits that the cited art does not teach or suggest a nucleic acid-lipid particle comprising a PEG-DAG conjugate and an amino lipid with an amino group having a pK_a of from 4 to 11. Applicant further submits that the cited art teaches away from the use of such amino lipids in lipid-nucleic acid particles. Additionally, Applicant submits that modification of the cited art to achieve inclusion of PEG-DAG conjugates in liposomes would be contrary to teachings of the cited art.

(i) Amino Lipids Comprising an Amino Group Having pK_a of from 4 to 11

The Office Action cites two references from Wheeler et al. (U.S. Patent No. 6,586,410; and corresponding PCT publication WO 96/40964), which disclose lipid-nucleic acid particles comprising cationic lipids. The cationic lipids taught for use by Wheeler et al. do not include the amino lipids called for by the presently amended claims. Further, Applicant submits that Wheeler et al. teach away from the use of amino lipids recited in the presently amended claims and thereby teach away from the presently claimed compositions.

At column 9, lines 11-14, Wheeler (i.e., the '410 patent) defines a cationic lipid as any lipid that carries a net positive charge at physiological pH, which includes the examples DODAC, DOTAP,

DDAB, DOTAP, DC-Chol and DMRIE. Amino lipids having an amino group with a pK_a of from 4 to 11, including the present exemplary titratable amino lipids DODAP and DODMA, are not embraced by the definition of cationic lipids set forth by Wheeler because they are not quaternary amines that have a permanent positive charge. Rather, these are titratable aminolipids which have a net positive charge only at acidic pH. For example, at physiological pH, substantially all DODAP and DODMA molecules are present as neutral molecules. As a result, complexed DNA that associates with DODAP or DODMA as a result of an interaction between these cationic lipids and anionic DNA is released at physiological pH.

Additionally, Wheeler teaches that a net positive charge at physiological pH is a functional requirement for cationic lipids useful in the DNA entrapment methods disclosed therein. In Example 28, Wheeler compares DODAC and DODAP and shows that when encapsulation is performed at a pH of 7.5, 75-80% entrapment is obtained with DODAC as compared to only about 5% with DODAP. Thus, DODAC and DODAP are taught not to be interchangeable, and Wheeler concludes: "This clearly demonstrates the requirement of positively charged lipids for DNA entrapment." Accordingly, Wheeler teaches away from the use of lipids which lack a net positive charge at physiological pH, including the aminolipids recited in the presently amended claims. Notably, the encapsulation mixtures disclosed by Wheeler do not include PEG-DAG.

The Office Action notes that DODMA is recited in claim 4 of Wheeler. However, the specification in Example 26 (column 50, line 18) exemplifies the use of DODMA-AN (N-[2, 3-(dioleyloxy)propyl]-N,N-dimethyl-N-cyanomethylammonium chloride), a lipid species distinct from DODMA that carries a net positive charge at physiological pH. Results using DODMA-AN are shown in Wheeler's Figure 41a and support that DODMA-AN carries a net positive charge at physiological pH. As Wheeler is clearly directed to the use of cationic lipids carrying net positive charge at physiological pH, a quality that is deemed to be required for function, the recitation of DODMA rather than DODMA-AN in the claims runs counter to the basis of the invention and appears to be an error.

Accordingly, Applicant submits that Wheeler does not teach or suggest the presently claimed nucleic acid-lipid particles. Further, the teachings of Cullis et al., Collins, and Unger et al., do not add to the disclosures of Wheeler et al. to arrive at the currently claimed nucleic acid-lipid particles and methods of using the same. Moreover, Applicant submits that Wheeler et al. teach away from the use of aminolipids recited in the presently amended claims, and thereby teach away from the presently claimed compositions.

(ii) PEG-DAG Conjugate

Wheeler et al. teach that nucleic acid particles may include PEG-lipids, such as PEG-ceramide.

Cullis et al. (U.S. Patent No. 6,417,326) disclose fusogenic liposomes comprising lipids capable of adopting a non-lamellar phase and reversibly associating with a bilayer stabilizing component. Suggested bilayer stabilizing components include PEG conjugated to ceramide.

Additional components suggested for use in the fusogenic liposomes include lipopeptides formed from diacylglycerol.

Collins (U.S. Patent No. 6,355,267) discloses methods for forming liposomes without the use of detergent or organic solvent which involve cycles of freezing and thawing, as well as liposomes so formed. One lipid suggested for use in the liposomes is diacylglycerol.

Unger et al. (U.S. Patent No. 6,143,276) disclose vesicles containing gaseous precursors. The use of DSPC in the preparation of lipid vesicles is disclosed.

At the time the invention was made, diacylglycerol was known to destabilize liposomes and to be useful in the preparation of fusogenic liposomes. As described in Basanez et al. (Biophysical Journal, 70: 2299-2306, 1996, attached as Exhibit A) even relatively low molar ratios of diacylglycerol (2%) can promote a phase transition from the lamellar to nonlamellar phases. These authors state (page 2305, lines 28-31) "[i]n conclusion, the above results provide clear examples of a lipid (diacylglycerol) that facilitates lamellar to nonlamellar phase transitions in two different lipid systems and promotes membrane fusion...". Further Kinnunen et al. (U.S. patent 6,074,667) disclose that diacylglycerol facilitates the fusion of liposomes with cell membranes (e.g., claims 4, 7 and 9) and refer to diacylglycerol as a "helper lipid" which has "fusogenic properties meaning that the lipid has the property of facilitating the fusion of lipid membranes" (column 3, lines 32-34).

Cullis et al. teach the inclusion of diacylglycerol containing lipopeptides in liposomes to promote liposome fusion with endosomal membranes following cellular uptake (column lines 25-30; column 16, lines 58-60), consistent with the destabilizing and fusion-promoting properties of diacylglycerol. Cullis et al. also teach the inclusion of PEG-ceramide conjugates in liposomes, however the PEG-ceramide conjugates are disclosed to serve as stabilizing agents. Accordingly, the use of diacylglycerol in place of ceramide in PEG conjugates, which the Office Action suggests would have been obvious, is not compatible with the teaching of Cullis et al., as it is contrary to the intended purpose of PEG conjugates and knowledge held in the art in respect of diacylglycerol.

Accordingly, at the time the present invention was made, there would not have been motivation to modify the teaching of Cullis et al. to use diacylglycerol in the form of a PEG conjugate. Additionally, Collins, Unger et al., and Wheeler et al. do not add to the disclosure of Cullis et al. to arrive at the currently claimed nucleic acid-lipid particles comprising PEG-DAG, and methods of using the same. Applicant submits that at the time the present invention was made diacylglycerol was not known in the art as a component that would be useful in the preparation of stable nucleic acid-lipid particles intended to protect an encapsulated polyanionic nucleic acid. Contrary to expectations, it was surprisingly discovered by the present inventors that when diacylglycerol is coupled to a hydrophilic polymer, PEG, the resulting conjugate can be included in stable liposomes that encapsulate and protect a nucleic acid.

In summary, the cited references, considered alone or in combination, do not teach or suggest a nucleic acid-lipid particle comprising a lipid layer surrounding and encapsulating a central region containing a polyanionic nucleic acid, wherein the lipid layer comprises (i) an amino lipid

comprising an amino group having a pK_a of from 4 to 11, and (ii) a PEG-DAG conjugate. Further, there would have been no motivation to modify the teachings of the cited art to achieve nucleic acid-lipid particles comprising PEG-DAG and amino lipids as presently recited. Indeed, such modifications would have been deterred by teachings in the art. Accordingly, Applicant submits that the currently claimed subject matter is not rendered obvious in view of the cited art, and respectfully requests withdrawal of the rejection and allowance of the claims.

Obviousness-type Double Patenting Rejections

As a preliminary matter, Applicant points out that the present application and U.S. Patent Nos. 6,287,591 and 6858,225 are each subject to an obligation of assignment to the University of British Columbia.

U.S. Patent No. 6,586,410

Claims 85-93, 97-106, and 108 stand rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-29 of U.S. Patent No. 6,586,410 in view of Collins (U.S. Patent No. 6,355,267) and Cullis et al. (U.S. Patent No. 6,417,326) and Wheeler et al. (WO 96/40964). Claims 94-96, and 107 stand rejected in further view of Unger (U.S. Patent No. 6,143,276). Applicant respectfully traverses the rejection.

For reasons discussed above, Applicant submits that the subject matter of the currently amended claims is not obvious in view of the claims of Wheeler et al., U.S. Patent No. 6,586,410, in further view of Collins, Cullis et al., and Wheeler et al., WO 96/40964. Applicant respectfully requests withdrawal of the rejection and allowance of the claims.

U.S. Patent No. 6,858,225

Claims 85-93, 97-106, and 108 stand rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-21 of U.S. Patent No. 6,858,225 in view of Collins (U.S. Patent No. 6,355,267) and Cullis et al. (U.S. Patent No. 6,417,326) and Wheeler et al. (WO 96/40964). Claims 94-96, and 107 stand rejected in further view of Unger et al. (U.S. Patent No. 6,143,276) and Wheeler et al. (U.S. Patent No. 6,586,410).

Upon notification that the application is otherwise in condition for allowance, Applicant will file an appropriate terminal disclaimer.

U.S. Patent No. 6,287,591

Claims 85-93, 97-106, and 108 stand rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-72 of U.S. Patent No. 6,287,591 in view of Collins (U.S. Patent No. 6,355,267) and Cullis et al. (U.S. Patent No. 6,417,326). Claims 95, 96, and 107 stand rejected in further view of Wheeler et al. (U.S. Patent No. 6,586,410).

The Office Action expresses that it would have been obvious to use DODMA as the aminolipid as suggested by Wheeler. Applicant reiterates the position that Wheeler et al. discloses the use of DODMA-AN, a composition different from DODMA and having properties distinct from

DODMA that are critical to the invention of Wheeler. Nevertheless, without admission, upon notification that the application is otherwise in condition for allowance, Applicant will file an appropriate terminal disclaimer.

U.S. Patent No. 6,734,171

Claims 85-93, 97-106, and 108 stand rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-19 of Saravolac et al. (U.S. Patent No. 6,734,171) in view of Collins (U.S. Patent No. 6,355,267) and Wheeler et al. (U.S. Patent No. 6,586,410). Claim 94 stands rejected in further view of Unger et al. (U.S. Patent No. 6,143,276). Claims 95, 96, and 10 stand rejected in further view of Wheeler et al. (U.S. Patent No. 6,586,410). Applicant respectfully traverses the rejection.

U.S. 6,734,171 and the present application are not commonly owned, as the '171 patent is owned by Inex Pharmaceuticals Corporation. Further, the priority date of the present application precedes that of U.S. 6,734,171. Applicant submits the present double patenting rejection is improper and respectfully requests withdrawal of the rejection and allowance of the claims.

CONCLUSION

Applicant respectfully submits that all pending claims of the present application satisfy the requirements for patentability and are in condition for allowance. Early indication of the same is therefore respectfully requested.

If a telephone call will help expedite any aspect of the prosecution of the instant application the Examiner is encouraged to contact the undersigned at 415-781-1989, or by facsimile at 415-398-3249.

Respectfully submitted,
DORSEY & WHITNEY LLP

Dated: 5/8/06

BY: Todd A. Lorenz, Reg. No. 39,754
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Customer Number: 32940
Dorsey & Whitney LLP
Intellectual Property Department
555 California Street, Suite 1000
San Francisco, Ca 94104-1513
Telephone: (415) 781-1989
Facsimile: (415) 398-3249

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Diacylglycerol and the Promotion of Lamellar-Hexagonal and Lamellar-Isotropic Phase Transitions in Lipids: Implications for Membrane Fusion

Gorka Basáñez, José Luis Nieva, Emilio Rivas, Alicia Alonso, and Félix M. Goñi
Grupo Biomembranas, Unidad Asociada al C.S.I.C., Departamento de Bioquímica, Universidad del País Vasco, 48080 Bilbao, Spain

ABSTRACT Changes in steady-state fluorescence anisotropy of 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) are applied to the detection of lamellar-hexagonal transitions in egg phosphatidylethanolamine. Even low (2 mole%) proportions of diacylglycerol decrease the hexagonal transition temperature considerably, as confirmed by differential scanning calorimetry. Diacylglycerol is also found to promote a lamellar to "isotropic" (Q^{224} cubic) transition in mixtures of phosphatidylcholine:phosphatidylethanolamine:cholesterol. This nonreversible transition is also observed by ^{31}P nuclear magnetic resonance and detected as a large increase in TMA-DPH steady-state anisotropy. The same technique reveals as well that lysophosphatidylcholine counteracts the effect of diacylglycerol and stabilizes the lamellar phase in both transitions. Diacylglycerol and lysophosphatidylcholine are known to respectively promote and inhibit membrane fusion in a variety of systems. These data are interpreted in support of the hypothesis of a highly bent structural fusion intermediate ("stalk"). They also show the interest of lipid-phase studies in predicting and rationalizing membrane fusion mechanisms.

INTRODUCTION

The well-established concept of a lipid bilayer as the structural basis of cell membranes is nonetheless compatible with the idea of transient nonbilayer structures playing important roles in various aspects of cell physiology. In particular, lipid structures of the kind represented by the so-called hexagonal II phase and the various cubic phases (Luzzati, 1968) have been invoked to explain various membrane phenomena, e.g., ion transport by membrane proteins (Cheng et al., 1986), intercell contacts (Hein et al., 1992), or membrane fusion (Seddon, 1990; Walter et al., 1994; Chernomordik and Zimmerberg, 1995; Chernomordik et al., 1995a,b). In turn, the involvement of nonlamellar structures in cell physiology or cell pathology processes has given rise to a renewed interest in lamellar to nonlamellar phase transitions in model systems (Hong et al., 1988; Epand et al., 1988; Siegel et al., 1989; van Langen et al., 1989; Seddon, 1990; Chen and Cheng, 1990; Allen et al., 1990; Han and Gross, 1992; Yao et al., 1992; Epand et al., 1992; Luzzati et al., 1993; Ambrosini et al., 1994; Siegel et al., 1994; Koynova and Caffrey, 1994; Goldberg et al., 1994).

Previous studies from this laboratory have described a model system for membrane fusion in which phospholipase C promotes fusion of large unilamellar liposomes via the in situ generation of diacylglycerol (Nieva et al., 1989, 1993, 1995; Burger et al., 1991; Goñi et al., 1994). This system, which has been further explored by other authors for the case of small unilamellar vesicles (Luk et al., 1993), appears to be the only fusion model in which fusion is driven by a catalytic agent. Under our conditions, optimum fusion is observed with liposomes made of PC:PE:CH (2:1:1, molar ratio) (Nieva et al., 1989). Recent nuclear magnetic resonance (NMR) and x-ray diffraction data (Nieva et al., 1995) suggest that, although the above lipid mixture gives rise to stable lamellae, the substitution of 5–10% of phospholipids by diacylglycerol greatly facilitates the formation of H_{II} hexagonal or cubic phases.

In view of these observations, a series of experiments has been carried out in our laboratory exploring the lamellar to nonlamellar transitions of pure phosphatidylethanolamine (PE) and PC:PE:CH mixtures by means of changes in fluorescence anisotropy of DPH-containing probes, mainly 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH).The latter probe has been extensively used to detect lipid phase transitions (van Langen et al., 1989; Cheng, 1990; Ambrosini et al., 1994), and we have demonstrated its application to monitoring the thermotropic transition from the lamellar to the H_{II} hexagonal phase in an unsaturated PE, and the requirement of vesicle aggregation for the transition to take place (Nieva et al., 1990). In the present paper we extend the application of TMA-DPH to the detection of lamellar-isotropic (cubic) phase transitions, and we report on our observations of the ability of diacylglycerol to facilitate lamellar to nonlamellar transitions in pure PE and in PC:PE:CH mixtures, supporting the proposed mechanism for phospholipase C-promoted liposome fusion. Occasionally, ^{31}P-NMR and differential scanning calorimetry (DSC) have been used in support of our fluorescence measurements. Moreover, lysolecithin has been shown to inhibit fusion in our system (Nieva et al., 1993), as well as in others (Chernomordik et al., 1993, 1995a); the fluorescence anisotropy data indicate that this lipid stabilizes the lamellar phase in systems containing PE, thus opposing the effect of diacylglycerols.

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Address reprint requests to Dr. Alicia Alonso, Departamento de Bioquímica, Universidad del Pais Vasco, Apartado 644, 48080 Bilbao, Spain. Tel.: 34-4-4647700, ext. 2407; Fax: +34-4-4648500; E-mail: gbpaliza@lg.ehu.es.

Dr. Rivas's permanent address is Instituto de Biología Celular, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina.

0006-3495/96/05/2299/08 $2.00

EXHIBIT A

MATERIALS AND METHODS

Egg phosphatidylcholine (PC), egg PE, and a 1,2-diacylglycerol derived from egg PC were grade I from Lipid Products (South Nutfield, England); egg lysolecithin was from Avanti Polar Lipids (Alabaster, AL); cholesterol was from Sigma (St. Louis, MO). Diacylglycerols contained a trace of the 1,3-isomer. All lipids were used without further purification. DPH, PC-DPH, and TMA-DPH were from Molecular Probes (Eugene, OR).

Lipids and probe were mixed at a 500:1 ratio in organic solvent; then the solvent was evaporated and the mixture was vacuum-dried for at least 2 h in the dark. In mixtures containing PC:PE:CH (2:1:1 mol ratio) and diacylglycerol or lysolecithin, the latter components are substituting for part of the phospholipid, so the composition of a mixture with, e.g., 10% diacylglycerol is PC:PE:CH:diacylglycerol (43:22:25:10). Multilamellar PE vesicles were formed in borate buffer (5 mM borate, 150 mM NaCl, 0.1 mM EDTA, pH 9.5) at room temperature and pelleted by centrifugation in a bench-top centrifuge for the spectroscopic or calorimetric studies. When required, PE liposomes were dialyzed against citrate buffer (10 mM citrate, 150 mM NaCl, 0.1 mM EDTA, pH 5.0) at 15°C, which induces liposome aggregation. Vesicles based on PC:PE:CH mixtures were prepared in HEPES buffer (10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.0) and pelleted by centrifugation for spectroscopic analysis.

Steady-state fluorescence anisotropy was recorded in a MPF-66 Perkin-Elmer fluorometer. TMA-DPH fluorescence was excited at 360 nm; emission was recorded at 430 nm. Lipid dispersions were examined using a frontal disposition, in a thermostated assembly consisting of two microscope cover slides cut to fit diagonally a standard 1 × 1 cm fluorescence cuvette filled with water. The pelleted or aggregated lipid was located between the slides, and the four sides were tightly sealed with plasticine (Nieva et al., 1990). A 390-nm cutoff filter was used to prevent scattered light from reaching the detector. The expression used to calculate anisotropy (R) is described by Lakowicz (1983). Samples were heated at 10°C/h. For PE-based samples, data from the first run were neglected; otherwise, subsequent runs gave similar results. In the case of PC:PE:CH mixtures with diacylglycerol, the heating effects were irreversible; thus only one heating run was performed.

Differential scanning calorimetry was performed in a Perkin-Elmer DSC-2C calorimeter operating in the low-temperature mode, with solid CO_2/methanol as a coolant, with a heating or cooling rate of 1.25 or 5°C/min. The aggregated lipid (~10 μl) was enclosed in Perkin-Elmer aluminum "volatile" sample pans. At least two runs were performed on each sample, neglecting the data from the first run.

^{31}P-NMR spectra were recorded in a KM360 Varian spectrometer operating at 300 MHz for protons. Spectral parameters were 45° pulses (10 μs), pulse interval 3s, sweep width 16 kHz, full proton decoupling. One thousand FID were routinely accumulated from each sample; the spectra were plotted with a line broadening of 80 Hz. Samples were equilibrated for 10 min at each temperature before data acquisition.

RESULTS

Phase transitions in pure PE

Changes in anisotropy of DPH, PC-DPH, and TMA-DPH dispersed in egg PE at pH 5.0 (i.e., in the form of lipid aggregates) are shown in Fig. 1 *A* as a function of temperature. All three probes detect a transition starting near 10°C, corresponding to a hydrocarbon chain order-disorder conformational transition (Allen et al., 1990; Nieva et al., 1990). In addition, PC-DPH and TMA-DPH, but not DPH, detect a second transition whose onset is located at ~30°C. The latter change corresponds to a lamellar (L_α) to hexagonal (H_{II}) phase transition (Seddon, 1990; Allen et al., 1990; Cheng, 1990; Nieva et al., 1990; Castresana et al., 1992). There is a wide variation in the published onset temperatures of this transition (T_h), because of the natural variability of the lipid fatty acid composition. Chen and Cheng (1990) have shown the advantages of PC-DPH in the study of this system by time-resolved fluorescence anisotropy, but it is clear from Fig. 1 *A* that the largest signal is obtained from TMA-DPH in the steady state, so that this probe was used in all further experiments. As discussed in our previous paper (Nieva et al., 1990), for the L_α-H_{II} transition to occur, lipid vesicles must first be aggregated, e.g., by dialysis against pH 5.0 buffer. This is seen in Fig. 1 *B*, in which the thermotropic effects on TMA-DPH anisotropy are comparatively shown at pH 5.0 and pH 9.5. The L_α-H_{II} transition is not observed at the higher pH because PE is negatively charged under such conditions, and aggregation is hindered by electrostatic forces (Allen et al., 1990).

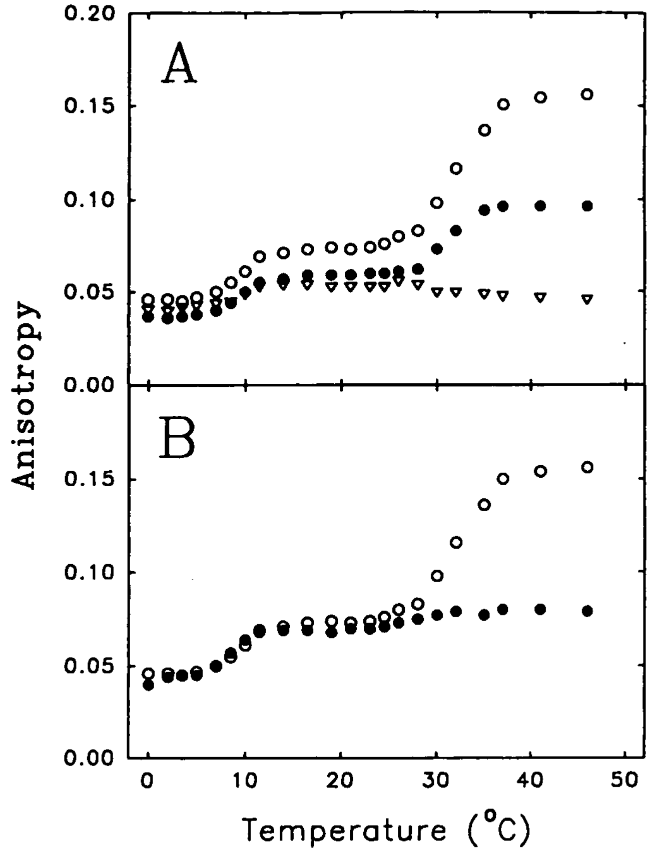

FIGURE 1 Thermotropic phase transitions of egg PE as detected by changes in anisotropy of diphenylhexatriene-containing probes. (*A*) A comparative study of three probes: ▽, DPH; ●, PC-DPH; ○, TMA-DPH. (*B*) Effect of pH on the L_α-H_{II} transition. Probe: TMA-DPH. ○, pH 5.0; ●, pH 9.5.

The frontal disposition technique used in our fluorescence measurements requires the lipid aggregates to be disposed in a sandwich structure between two microscope coverslips. Preliminary observations showed that the anisotropy measurements may vary with the thickness of the lipid layer. For that reason, a systematic study was performed in which the thickness of the lipid sandwich was estimated from its turbidity (absorbance at 550 nm) when located in

the sample holder of a dual-beam spectrophotometer against pure water. Measurements of TMA-DPH anisotropy were performed at 5, 25, and 50°C (temperatures at which L_β, L_α, and H_{II}, respectively, should predominate), for A_{550} values between ~0.15 and 1.1. The results in Fig. 2 show that there is indeed an important variation in the relative anisotropies for preparations with $A_{550} < 0.6$. Furthermore, at very low turbidities the gel to liquid-crystalline transition may go undetected, if it is not marked by an apparent decrease in steady-state anisotropy. The previous data for the L_α-H_{II} transition as detected by TMA-DPH indicate a decline in the order parameter (Cheng, 1990), in agreement with an increased anisotropy, as seen for preparations with $A_{550} >$ 0.5. All of our anisotropy studies have been carried out with samples of $A_{550} > 0.8$. The problem of the influence of sample turbidity on TMA-DPH anisotropy has already been described by Storch et al. (1989) for a system involving whole cells.

Effects of diacylglycerol

The effect of diacylglycerol on the thermotropic PE transitions as detected through TMA-DPH anisotropy is shown in Fig. 3. The L_β-L_α transition appears to be largely unaffected, whereas the L_α-H_{II} transition is shifted to lower temperatures as the diacylglycerol concentration is increased. The effect of this lipid is already appreciable at 2% (mol:mol). With 10% diacylglycerol, a single transition is observed starting below 10°C and consisting probably of the simultaneous L_β-L_α and L_α-H_{II} transitions. Chen and Cheng (1990) have made similar observations, including the merging of both transitions, by infrared spectroscopy.

These phenomena can be better understood by looking at the phase transitions by differential scanning calorimetry (Fig. 4).

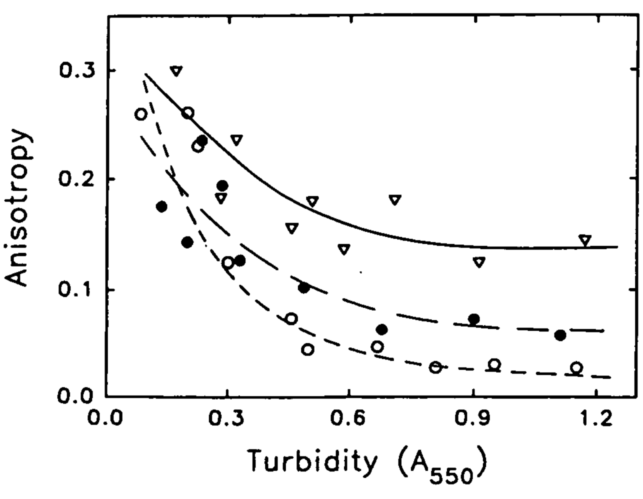

FIGURE 2 Effect of lipid film thickness on the apparent anisotropy of TMA-DPH in egg PE (pH 5.0). Thickness is measured as turbidity (A_{550}) in a spectrophotometer, as detailed under Materials and Methods. Anisotropies are measured at 5°C (○), 25°C (●), and 50°C (▽), temperatures at which L_β, L_α, and H_{II} should, respectively, predominate. The curves shown are only meant to guide the eye and do not have any theoretical foundation.

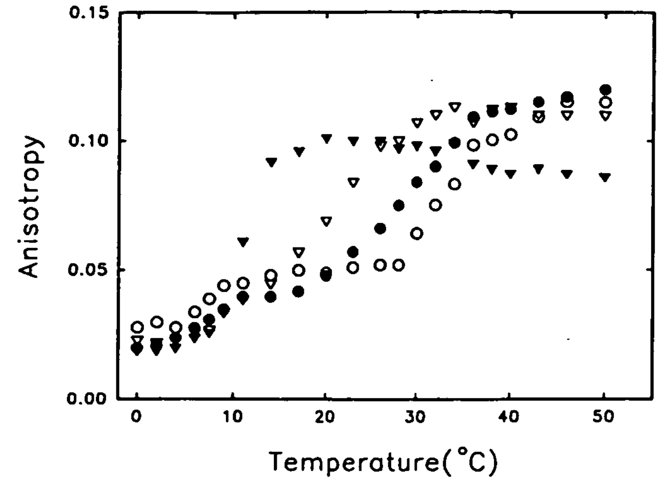

FIGURE 3 Destabilization of the L_α lamellar phase of PE by diacylglycerol. Thermotropic phase transitions are detected through changes in TMA-DPH anisotropy, pH 5.0. Diacylglycerol concentration (mol%): ○, 0%; ●, 2%; ▽, 5%; ▼, 10%.

Again the lipids are aggregated by dialysis versus a pH 5 buffer and encapsulated in gas-tight vials. The thermograms (Fig. 4 *A*) clearly show both transitions; the L_α-H_{II} has a much smaller molar enthalpy change (smaller area under the peak). The midpoint temperature of the L_β-L_α transition (T_m) increases slightly (Fig. 4 *B*), whereas the corresponding value for the hexagonal transition is clearly depressed. Ortiz et al. (1988) showed that diacylglycerol increased the T_m of dipalmitoyl PC, and Epand et al. (1988) detected a decrease in the L_α-H_{II} transition temperature of dielaidoyl PE. Diacylglycerol also makes the L_α-H_{II} transition less cooperative, as indicated by the increased width of the corresponding endotherm (Fig. 4 *A*). The thermogram corresponding to the sample with 10% diacylglycerol is interesting, because it shows unequivocally that both transitions occur and that the early part of the L_α-H_{II} transition occurs simultaneously with the final part of the L_β-L_α melting (see on this point Katsaras et al., 1993).

In a different series of experiments the effect of diacylglycerol on the phase behavior of PC:PE:CH (2:1:1 mol ratio) mixtures was studied. Liposomes with this composition have been found to fuse with each other when diacylglycerol is generated in situ by the action of phospholipase C (Nieva et al., 1989). TMA-DPH anisotropy does not display any abrupt change by heating the PC:PE:CH (2:1:1) mixture from 20 to 80°C (Fig. 5 *A*), in agreement with previous observations on its stability (Nieva et al., 1995). However, in the presence of 5% DG, a transition is detected starting at ~55°C. The nature of this transition is made clear by the ^{31}P-NMR spectra shown in Fig. 5 *B*. In the absence of diacylglycerol, the mixture PC:PE:CH (2:1:1) shows the asymmetric lineshape, with a shoulder at the lower field side, that is characteristic of the lamellar phase (Seelig, 1978). However, the presence of 5% diacylglycerol in the sample produces, above 50°C, the formation of an isotropic phase that becomes predominant at ~70°C and beyond. The

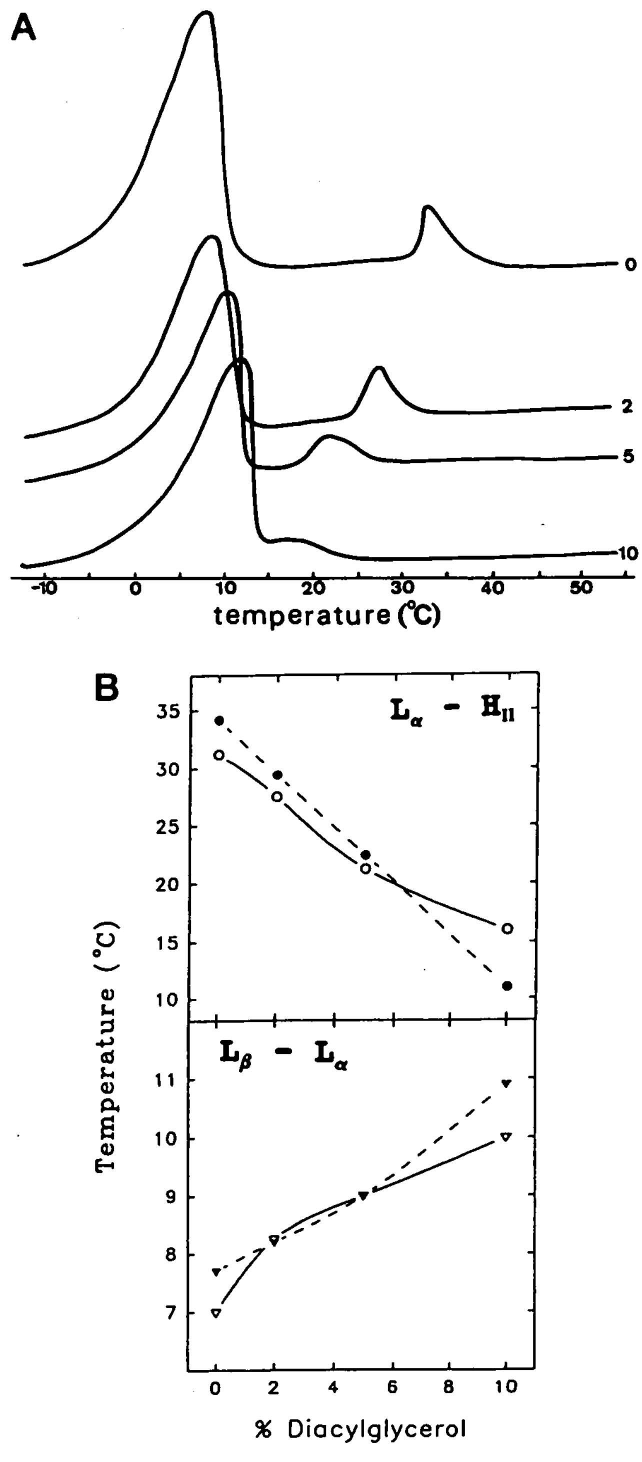

FIGURE 4 Thermotropic phase transitions of egg PE as detected by differential scanning calorimetry, and the effect of diacylglycerol. (*A*) DSC thermograms. The low- and high-temperature endotherms correspond, respectively, to the L_β-L_α and L_α-H_{II} transitions. The diacylglycerol concentration (mol%) is indicated by each curve. The calorimetric pans contained similar amounts of lipid. (*B*) Change in transition temperatures produced by diacylglycerol. Circles: T_m of the L_α-H_{II} transition. Triangles: T_m of the L_α-L_β transition. Open symbols: data from DSC thermograms. Filled symbols: data from TMA-DPH anisotropy. Note the different scales in the top and bottom of *B*.

nature of this isotropic phase cannot be further determined from the ^{31}P-NMR data, but our previous x-ray diffraction studies suggest that it is a cubic phase (Q^{224}) (Nieva et al., 1995). Thus the increase in TMA-DPH anisotropy observed in the sample containing 5% diacylglycerol appears to be due not to a L_α-H_{II} transition but to a lamellar-cubic transition. (The present data do not allow further speculation on, e.g., intermediate steps between the L_α and Q_{224} phases.) One important point on this transition is that it is virtually irreversible; as the system is cooled back down to room temperature, the anisotropy does not decrease (Fig. 5 *A*) and the NMR signal remains isotropic (data not shown). Previous studies of PC:PE:CH mixtures with diacylglycerol by ^{31}P-NMR and x-ray diffraction have shown that the isotropic (cubic) phase is stable for days after the samples are annealed at room temperature, and chemical analysis indicates that the thermal treatments described here do not lead to any detectable degradation of the lipids under study (Nieva et al., 1995; Nieva et al., unpublished observations). Israelachvili's theory of molecular shapes of lipidic membrane components (Israelachvili et al., 1980) predicts that lipid molecules of "conical" shape, e.g., PE or diacylglycerol (lipids that have a negative spontaneous curvature, in the nomenclature of Helfrich, 1973), will favor the formation of inverted hexagonal (H_{II}) structures, whereas lipids of "inverted conical" shape (or positive curvature), e.g., lysophospholipids, will counteract the latter tendency. Because we found that PE was essential for fusion to occur in our PC:PE:CH system in the presence of phospholipase C (Nieva et al., 1989), we tried successfully to confirm the inhibitory role of lysophosphatidylcholine (Nieva et al., 1993). To establish on molecular grounds this inhibitory effect, we have tested the effect of lysolecithin on the two cases of lamellar to nonlamellar transition studied above. For the L_α-H_{II} transition of PE, the data in Fig. 6 indicate that 10% lysophospholipid completely abolishes this transition, as seen both by changes in TMA-DPH anisotropy (Fig. 6 *A*) or by differential scanning calorimetry (Fig. 6 *B*). Note that both techniques show that the L_β-L_α transition remains unaltered.

In a similar way, TMA-DPH fluorescence anisotropy data reveal that lysolecithin abolishes the lamellar to isotropic transition brought about by diacylglycerol in the PC:PE:CH (2:1:1) mixture (Fig. 7). ^{31}P-NMR spectra (not shown) confirm the presence of a lamellar phase between 20 and 80°C for PC:PE:CH:diacylglycerol:lysophospholipid (47:23:25:5:10, mole ratio).

DISCUSSION

The above results deserve some discussion at least from two points of view, the mechanism by which diacylglycerol facilitates the phase transitions, and the implications for cell membrane fusion. In addition, there is a methodological aspect that raises some questions, and we shall discuss this first.

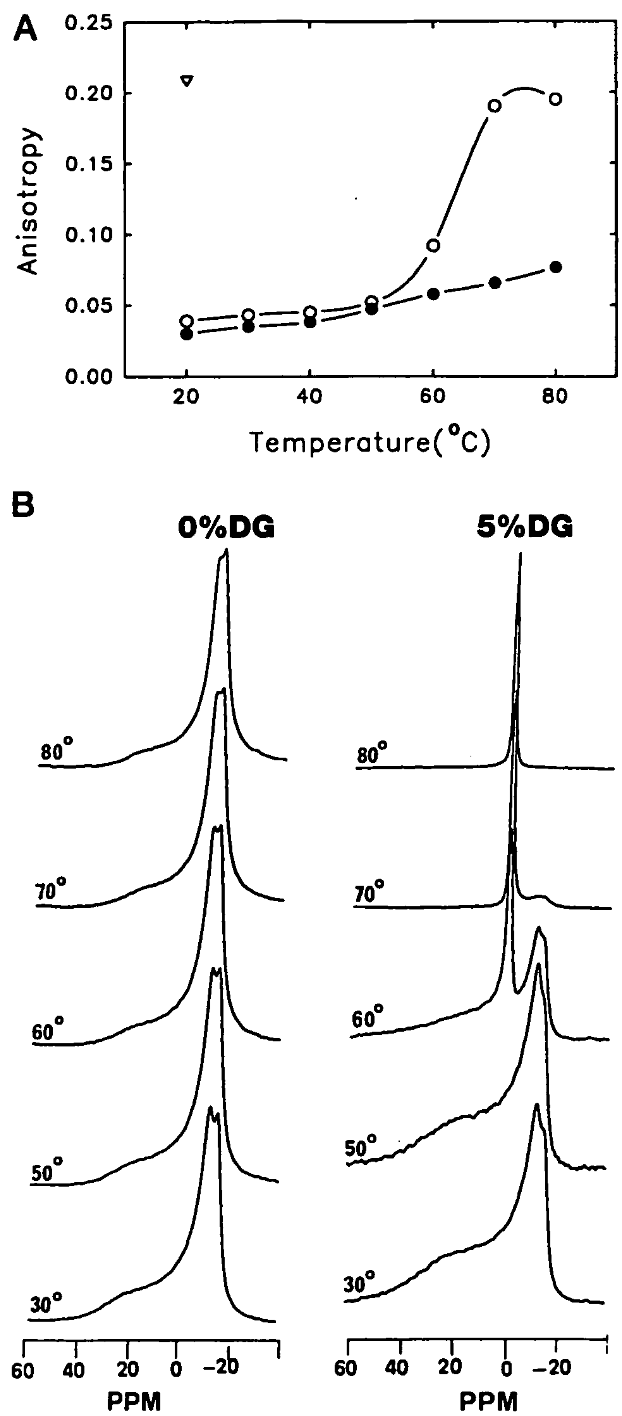

FIGURE 5 Phase transitions in the mixture PC:PE:CH (2:2:1 mol ratio). (*A*) Anisotropy of TMA-DPH, pH 7.0. ●, 0%; ○, 5% diacylglycerol. ▽, Sample containing 5% diacylglycerol, heated to 80°C and cooled back to 20°C. (*B*) ^{31}P-NMR spectra, with and without 5% diacylglycerol, recorded at various temperatures.

Changes in fluorescence anisotropy

The experimental basis of this paper is the observation of an increase in TMA-DPH fluorescence anisotropy upon the lamellar to nonlamellar thermotropic transition in two lipid systems. One of them, unsaturated PE of natural origin, has been studied in detail, and the L_α-H_{II} transition has been characterized by x-ray diffraction (Luzzati, 1968; Seddon, 1990; Castresana et al., 1992), ^{31}P-NMR (Cullis and de Kruijff, 1978), infrared spectroscopy (Mantsch et al., 1981), and differential scanning calorimetry (Gawrisch et al., 1992), among other techniques. The second system, a complex mixture of PC, PE, CH, and diacylglycerol, has not been characterized until recently (Nieva et al., 1995); it appears to undergo a lamellar to isotropic transition, where the isotropic component is a Q^{224} cubic phase. Lamellar-hexagonal transitions have been monitored with a variety of fluorescence spectroscopy techniques. In particular, the case of PE and TMA-DPH has been studied in detail by Cheng and co-workers (van Langen et al., 1989; Cheng, 1990), using angle-resolved and time-resolved fluorescence. According to their data, the local order parameter of TMA-DPH in the H_{II} phase is about 25% higher than that in the L_α phase, and the rotational diffusion constant of the probe decreases with the L_α-H_{II} transition. These data are compatible with the observed increase in steady-state anisotropy (Fig. 1). No such detailed studies have been carried out, to

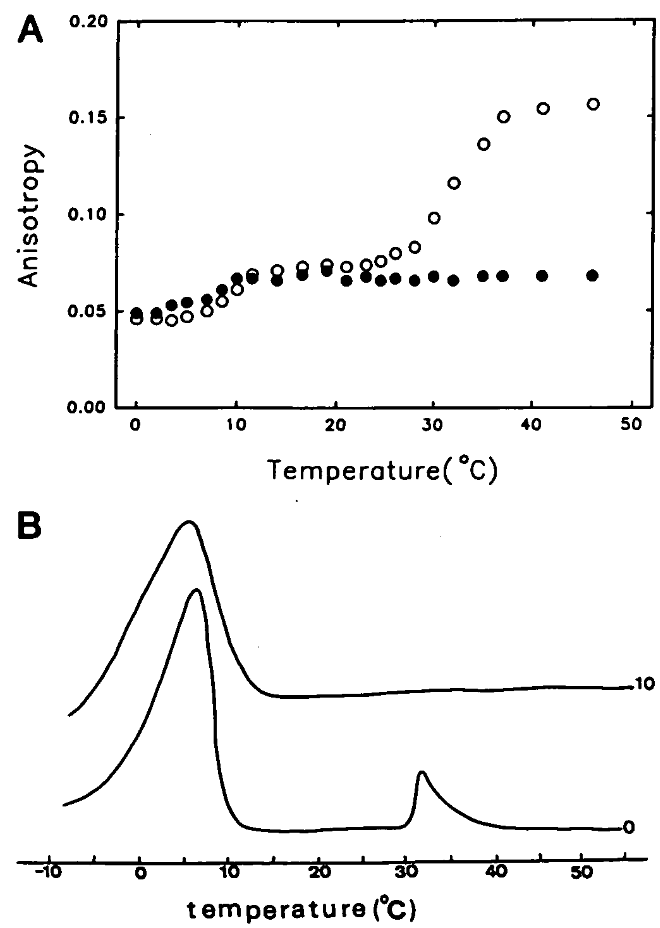

FIGURE 6 Stabilizing effect of lysophosphatidylcholine on the L_α-H_{II} transition of egg PE. (*A*) Anisotropies of TMA-DPH, pH 5.0. ○, Control; ●, +10% lysophospholipid. (*B*) DSC thermograms: 0 and 10% lysophospholipid, as indicated by each curve. The calorimetric pans contained approximately similar amounts of lipid.

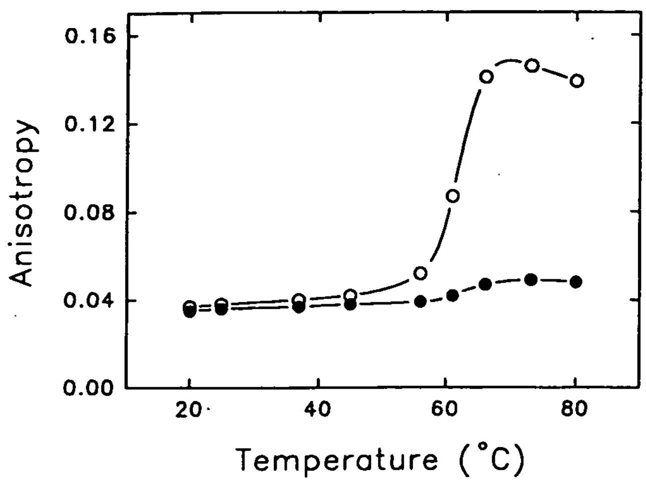

FIGURE 7 Stabilizing effect of lysophosphatidylcholine on the L_α-Q^{224} transition of the mixture PC:PE:CH:diacylglycerol (47:23:25:5). ○, Control; ●, +10% lysophospholipid.

the authors' knowledge, with fluorescent probes in cubic phases; thus the observed increase in TMA-DPH anisotropy that accompanies the lamellar-isotropic transition of PC:PE: CH:diacylglycerol (Fig. 5 *A*) remains to be explained on firm spectroscopic grounds. It should be noted that cubic phases are noted by their high viscosity, much higher than that of the lamellar or hexagonal phases (Luzzati et al., 1993); this property would be compatible with an increased anisotropy as seen in Fig. 5 *A*. The change in anisotropy (ΔR) between fluid lamellar and cubic phases (Fig. 5 *A*) is much larger than the one observed between fluid lamellar and hexagonal phases in Fig. 1 *A* (~0.16 and ~0.08, respectively). The possibility of detecting a lamellar-cubic transition with the relatively simple fluorescence polarization equipment is also important methodologically speaking, particularly when the small enthalpy change associated with such a transition makes difficult its detection by differential scanning calorimetry (Siegel and Banschbach, 1990).

The properties of diacylglycerol

Relatively small proportions of diacylglycerol (2–5 mol%) have marked effects in facilitating lamellar to hexagonal or lamellar to cubic transitions. The formation of nonbilayer lipid phases by diacylglycerol/phospholipid mixtures has been described previously (Goldberg et al., 1994, and references therein). Diacylglycerol may act in at least two ways: by facilitating intermembrane contacts and/or increasing the lipid surface curvature. Intermembrane contacts are required in transitions from lamellar to hexagonal or cubic phases because they imply changes from an essentially one-dimensional to two- or three-dimensional structures. The requirement of vesicle aggregation for a L_α-H_{II} transition to occur was pointed out previously (Allen et al., 1990; Nieva et al., 1990), and it is probably valid as well for lamellar to cubic transitions. In our studies on phospholipase C-promoted vesicle aggregation and fusion (Nieva et al., 1993), we found that diacylglycerol has the effect of increasing vesicle-vesicle aggregation. This can be understood by the relative dehydration brought about by the presence of diacylglycerol in the bilayer surface—thus the partial removal of the major barrier to close contact of phospholipid bilayers (Rand, 1981). L_α-H_{II} transitions are accompanied by dehydration (Cheng, 1990; Castresana et al., 1992; Katsaras et al., 1993; López-García et al., 1994), thus the facilitating role of diacylglycerol is easy to understand. (See also Kozlov et al. (1994) for a discussion of competing bending and hydration energies in a complex case of hexagonal-lamellar-hexagonal phase transitions.) However, Das and Rand (1986) found that removal of up to 18% of PC polar headgroups did not result in closer apposition of the corresponding bilayers, so that the role of diacylglycerol in helping intermembrane contacts, particularly when it is symmetrically distributed in the bilayer, as in the present studies, is debatable.

More clear is the participation of this hydrophobic lipid in helping the formation of structures requiring high-curvature monolayers ("negative curvature"; Helfrich, 1973), as in the case of H_{II} or Q^{224} phases. Diacylglycerol increases the relative volume of the hydrocarbon, because unsubstituted glycerol headgroups partition preferentially in the hydrocarbon regions (Gulik et al., 1988); thus it tends to increase the monolayer negative curvature, as discussed previously (Luzzati, 1968; Israelachvili et al., 1980; Das and Rand, 1986; Chernomordik and Zimmerberg, 1995).

Implications for cell membrane fusion

The role of lipids in biological membrane fusion has recently been the object of two important reviews (Chernomordik et al., 1995b; Chernomordik and Zimmerberg, 1995). These authors have proposed that a common mechanism of lipid bilayer rearrangement underlies the various physiological phenomena of membrane fusion. The results in this paper may be considered in the light of those considerations. Essentially, the rearrangement of two lipid bilayers into a single one must involve the bending of the membranes. Most recent data in the literature suggest the existence of a strongly bent intermediate, the "stalk" (Chernomordik et al., 1987; Siegel, 1993), a transient structure formed by the contacting monolayers of two membranes.

The stalk has a net negative curvature (the curvature of a monolayer in the H_{II} phase is defined to be negative; Helfrich, 1973). Thus, including in the monolayer lipids that support formation of the H_{II} phase, will make the monolayer curvature more negative and promote stalk formation. The opposite effect will be found with lipids with a spontaneous positive curvature, i.e., micelle-forming lipids. The observations in this paper on the facilitating and inhibiting effects, respectively, of diacylglycerol and lysolecithin on lamellar to nonlamellar phase transitions explain very well,

through the stalk hypothesis, their respective effects on phospholipase C-promoted membrane fusion (Nieva et al., 1993, 1995). In more general terms, the stalk may be the "structural intermediate" that we proposed earlier (Nieva et al., 1993) and for which we suggest a structure related to a bicontinuous cubic phase (Nieva et al., 1995). Relevant to this point are the observations of Landh (1995) of cubic membranes as subcellular space organizers and the suggestion by Oberhauser et al. (1992) on the role of lipids in the exocytotic fusion pore.

The original stalk model was modified by Siegel (1993) to include the contribution of hydrophobic void spaces within the intermediate. Such void spaces could considerably increase the stalk energy. According to this idea the presence of small amounts of "impurities" in the form of neutral lipids could fill in the voids, thus decreasing energy and promoting stalk formation. This may be an additional mechanism of action for the facilitating role of diacylglycerol in lamellar to nonlamellar transitions and in liposome fusion, apart from those suggested above. It should be noted in this respect that diacylglycerol decreases the optimum temperature for liposome fusion in the presence of phospholipase C (Nieva et al., 1993). Furthermore, Walter et al. (1994) interpreted within this theoretical framework their observation that diacylglycerol increases divalent cation-induced lipid mixing between phosphatidylserine liposomes.

In conclusion, the above results provide clear examples of a lipid (diacylglycerol) that facilitates lamellar to nonlamellar phase transitions in two different lipid systems and promotes membrane fusion in different model systems and, conversely, of a lipid (lysolecithin) that inhibits such phase transitions and suppresses various fusion processes. These observations provide strong support for the hypothesis of the involvement of transient nonlamellar intermediates in the fusion process, and in particular for the so-called stalk hypothesis (Chernomordik et al., 1987; Siegel, 1993). The structure of the Q^{224} cubic phase, consisting of rods rather than micelles (Luzzati et al., 1993), that is formed under our conditions does not favor the putative "inverted micellar intermediates" as the structural fusion intermediates, in agreement with the observations by Siegel et al. (1994). See also on this point Nieva et al. (1995). However, a note of caution should be added at this stage, to dispel sketchy ideas on the dynamics of lipidic structures. The thermodynamic concept of phase should not be confused with the biological concept of structure. And the experimental conditions in which lipid phase transitions are studied differ markedly from those in which membrane fusion takes place, even in vitro. In practical terms, phases and phase structures are but idealizations of real biological structures. The structural fusion intermediate (be it a stalk or otherwise) is not or does not consist of a hexagonal or a cubic phase; it is instead a (hopefully) real though transient structure, in which the relative disposition of a population of lipid molecules resembles the ideal geometry of a hexagonal or a cubic phase. Of course this is not meant to dismiss the biological relevance of lipid phase studies; on the contrary, as shown in this paper, they constitute prime tools of diagnostic and predictive value for biophysical studies of membrane fusion.

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